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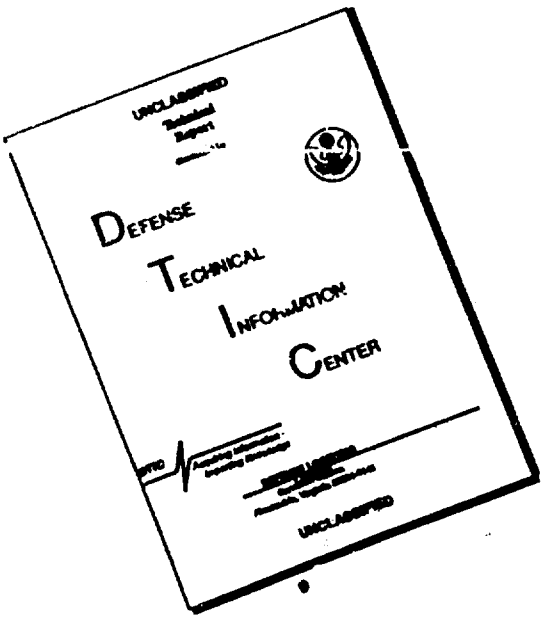

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Studies on Some Factors Influencing the Reliability of Botuline and Tetanus Anatoxin Titers According to the Flocculation Reaction

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The problem of a possible determination of antigenic properties of toxins and anatoxins by simple, quick and economical serological methods has attracted attention of investigators for a long time. Yet, in spite of very many reports written on the subject, the serological methods and, particularly, the flocculation reaction have not yet encountered any broad practical acceptance in development of tetanic and botuline preparations. This can be explained by variances occurring in the results from the titration of serums and anatoxins in vivo and in vitro, as well as by a lack of unified opinion pertinently to the nature and mechanism of serological reactions.

Only a few authors who studied the flocculation phenomenon (RAMON, GLENNY, KRESTOVNIKOVA and GOROKHOVNIKOVA, PAPPENHEIMER) assumed that the formation of visible flocculant is conditioned by reaction between the toxin and anatoxin. On the other hand, many investigators (BRONFENBRENNER and REICHERT, ZINGHER, EISLER and KOVACS, et al.) explained this by reciprocal action between the exclusively bacterial precipitinogens present in a toxin and bacterial precipitins that form concurrently with the antitoxin

during immunization processes.

Contradictory opinions also exist about the question whether the flocculation reaction is a true index of the antigenic value with regard to tetanic and botuline anatoxins. According to conclusions of RAMON, PEIGIGINA, VOLKOVA, VOROBEV, KOLESNIKOVA and SOKOLOV, the antigenic properties of botuline and tetanic anatoxins are directly proportional to the flocculation activity. BRONFENBRENNER and REICHERT, also NIGG, KHOTTL et al., KOERBER, PICE, PALLISTER, etc. do not emphasize the same conformity.

Contradictory results obtained by various investigators in using the flocculation reaction are explained by a lack of standard flocculation serums (KHALYAPINA and NIKOLSKAYA, SHAIN). Every researcher uses arbitrarily selected serum to effect the reaction and, at the same time, the selection of serums for flocculation is made by empirical method out of dozens, even hundreds of therapeutic serums.

In studying a possible utilization of the flocculation reaction in order to determine antigenic properties of botuline toxins type A, B and C, also tetanic anatoxins, we attempted to clarify the reasons for incogruities found in many instances of flocculation titers of anatoxins and their antitoxin binding activities with the intent on finding objective criteria for selection of flocculation serums.

We determined the flocculation capacity of crude and concentrated tetanic and botuline anatoxins by using refining and concentration methods of combined dialysis and also the "diaferm-3" method with antitetanic and antibotuline horse serums (with titers

from 4,000 to 16,000 AE in 1 ml), diluting them to 100 AE (antitetanic serum), or to 1,000 AE (antibotuline serums) per 1 ml.

The method to resolve flocculation reaction comprised the following schedule: into a number of test tubes we poured serums in increasing amounts, beginning with 0.05 ml and successively with 0.1 ml interval during the preliminary experiment, then with 0.02 to 0.04 ml in the subsequent experiment (in order to obtain more precise results); we added 0.5 to 1 ml of anatoxin to each test tube (undiluted anatoxin in the preliminary experiment; diluted to 200 L_f-dose in subsequent test); we stirred the mixture of serum and anatoxin and then left it to incubate at 45°. Next, we recorded the time of the initial flocculation appearance (L_f). The antigen concentration (flocculation titer) was expressed in L_f in accordance with the following equation (MARKOVICH, KHAUSTOVA, CHEBOTAREVICH):

$$T = Vc \frac{y}{m} n,$$

where y is the antitoxic titer of serum; where Vc is the flocculation dose of serum, namely the volume of diluted serum in which initial flocculation was obtained; where m is the dilution of serum; where n is the dilution of anatoxin.

Our findings revealed that the minimum amount of anatoxin we could determine by the flocculation reaction was 50 to 100 L_f for botuline and 5 to 10 L_f for tetanic anatoxins.

The antitoxin binding capacity of anatoxins was determined by titration on white mice, according to the method of TARASEVICH'S State Control Institute, with the use of expanded control series

in experimental dose of the toxin (MARKOVICH and VOROBEV). The results were expressed in binding units (EC) computed as a percentage of 1 AE.

At the time of the selection of flocculation serums our attention was usually centered on clearness and rapidity of the flocculation in a single narrow zone. This, however, often proved to be insufficient. One should always take into account the existence of absolute quantitative correlations between the anatoxins' titers as revealed by L_f according to the flocculation reaction and by EC, according to the antitoxin's binding reaction. If the flocculation reaction is specific, the correlation of EC/L_f for each anatoxin should be equal to one unit, provided that 1 EC and 1 L_f are equal to 1 AE.

Out of 15 botuline and antitetanic serums we selected 5 as the fastest flocculating substances. The correlation of EC/L_f equal to one unit was obtained for one serum only (type A serum, series No.33) out of 15 anatoxin series used in the experiment (see Table 1).

The magnitude of EC/L_f in remaining serums varied considerably, mainly toward exaggeration of the flocculation reaction's results.

Using as an example antibotuline serums type A, series No.33 and 1, we tried to clarify the reasons why one serum (series No.33) induces the obtainment of reliable flocculation results from all anatoxins used in the experiment, while another serum (series No.1) produces exaggeration of the L_f value in other section of anatoxins.

In studying a possible determination of antigenic properties of botuline anatoxins according to the ring-precipitation reaction

Table 1

Distribution of Tetanus and Botuline Anatoxins of the A, B and C Types According to the Magnitude of the EC/L_f Ratio

Serum	Serial No. of serum	Quantity of tested series of anatoxins	Quantity of anatoxin series with diverse ratio of EC/L_f						
			0.1	0.3	0.4	0.5	0.8	1	1.2
Antibotuline, type A	1	42	1	9	3	10	5	13	1
Antibotuline, type A	33	15	-	-	-	--	-	15	-
Antibotuline, type B	2	24	-	3	4	6	4	6	1
Antibotuline, type C	3	28	2	2	9	4	3	6	2
Antitetanic	2,235	70	-	2	5	13	14	30	6

(VORONOVA), we concluded that reliable results can be obtained only when antibodies to the heat-resistant bacterial precipitinogens are absent in the serum, since they condition the group specificity of a strain. We obtained such type-specific precipitating serums after inoculation of rabbits with microbic cells of a strain of other serological group (according to agglutination reaction), but not of a strain used for the preparation of anatoxins.

We assumed that the dependability of the flocculation titers proceeds from the factor that in this reaction and in the precipitation reaction participate bacterial (group) precipitinogens - precipitins in addition to specific antigens and antibodies (anatoxin - antitoxin).

The investigations proved that flocculent antibotuline and antitetanic serums contained in addition to the serum series No.33 also agglutinates and group precipitins (1:8 and 1:16) in higher titers (1:2,000 and 1:5,000). As long as botuline anatoxins and serums of the A and B types have been prepared with the use of strains that contained usual group precipitinogens of microbic cell, we observed a cross flocculation between the serums and anatoxins of these types*). With depletion of the serum type A by anatoxin type B and, vice versa, type B serum by the type A anatoxin, we were able to obtain serums flocculent and free from group precipitins, but they fully preserved their antitoxic properties.

The freeing of type A serum, series No.1, from group precipitins was accomplished in the following way: we determined the opti-

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*) - Serums of A and B types were monovalent and contained no admixtures of antitoxin of other type.

mum quantity of serum and anatoxin B in the cross flocculation reaction, then we mixed equivalent quantities of serum and antigen and, after separation of precipitate, we placed the test tubes in a refrigerator; next day we separated the sediment by centrifuging action and we used the supraseditment fluid as the flocculent serum type A.

The investigations proved (see Table 2) that type A serum, series No.1, freed from nonspecific antibodies, flocculated with all anatoxins type A in a zone that agreed with their antitoxin's binding activity ($EC/L_f \approx 1$), while no such conformity was observed prior to depletion. It should be added that depleted serum flocculated considerably slower and, at the same time, a delicate and thin flocculate was formed.

We also obtained good results from type-specific, precipitated rabbit serums that contained no group precipitins; they coincided with the titration results on animals (see Table 3).

Following a mixing of botuline anatoxins types A and B with purely antibacterial precipitating serums that contained no antitoxin, the precipitate separated immediately after addition of anatoxin, i.e. simultaneously in all test tubes regardless of the quantity of serum.

Hence, we assume that the flocculation phenomenon, observed after mixing anatoxins with suitable antitoxic serums, is a combined effect that is conditioned by the reaction of antitoxin and by concurrent reactions of bacterial precipitates - precipitinogens.

Taking into account the effect of the purity of serum (presence of precipitins in it) on the flocculation reaction's titer, we de-

Table 2

Reaction Results from the Flocculation of Botulinus Anatoxins Type A with Type A Serum Series No.1 Prior and After Its Depletion by Anatoxin Type B

Serial No. of type A anatoxin	Quantity of EC in 1 ml	Flocculation titer of type B serum (L_f in 1 ml)	Flocculation titer of type A serum			
			Prior to depletion		After depletion	
			L_f	EC/L_f	L_f	EC/L_f
1,419	600	2,000	1,100	0.54	560	1.08
966	1,000	4,000	4,000	0.25	1,200	0.8
163	200	2,000	200 and 2,000	1 and 0.1	195	1
1,400	400	1,600	800	0.5	396	1
5/1	100	2,000	400	0.25	97	1.02
1,518	600	1,880	1,200	0.5	620	0.9

Table 3

Reaction Results from the Flocculation of Botuline Anatoxins Types A and B with Precipitated Rabbit Serums Type A (300 AE in 1 ml) and B (200 AE in 1 ml)

Anatoxin titer and serum	Serial No. of anatoxin	Quantity of EC in 1 ml	Titer after flocculation reaction			
			with precipitating, type-specific rabbit serum		with antitoxic horse serum	
			L_f	EC/L_f	L_f	EC/L_f
A	1,418	650	720	0.9	1,200	0.5
	1,460	500	520	0.9	800	0.6
	163	200	210	0.9	200 and 2,000	1 and 0.1
	1,427	400	380	1	800	0.5
	134	50	45	1.1	800	0.6
	5/1	100	105	1	400	0.25
B	1,440	660	600	1.1	860	0.7
	1,550	660	700	0.9	1,200	0.5
	1,555	660	700	0.9	800	0.7
	1,495	40	40	1	100	0.4

cided to examine the importance of the purity degree of anatoxin with relation to nonspecific ballast proteins and the products of decomposition of cells, as this affects the magnitude of the anatoxin's flocculation titer with a given serum. Figure 1 shows a change in the ratio of EC/L_f, relevant to 26 series of tetanic

a = Purification degree of anatoxins (EC/mg of common nitrogen).

Figure 1 - A change in the ratio of EC/L_f for tetanic anatoxins with relation to their degree of purity.

anatoxins concentrated by the same method and plotted against a degree of their purity (EC in 1 mg of common nitrogen). With the advanced degree of anatoxin's purity from 90 to 300 EC per 1 mg of common nitrogen, the ratio of EC/L_f increased gradually and approximated 1. Subsequently, the ratio remained constant and equalled 1 for anatoxins that attained the purity degree of 300 EC per 1 mg of common nitrogen, or higher. This rule has been recorded in connection with botuline anatoxins of the A, B and C types.

The discussed findings indicate that, in using a serum which contains bacterial precipitins, the flocculation titers, that agree with the results of the antitoxin's binding reaction, can be observed only when concentrated anatoxins are sufficiently purified.

In crude botuline anatoxins we encountered at least 10 times higher concentrations of group precipitinogens per 1 EC than in concentrated anatoxins per 1 EC. Thus, as a rule, we observed two flocculation zones in crude botuline anatoxins. The actual zone represented a smaller quantity of the flocculent serum; it appeared later than the false flocculation, which along with its appearance involved a greater quantity of serum.

The investigation of the flocculent and antitoxin binding activities of crude botuline anatoxins of the A, B and C types /obtained from cultures of diverse growth periods (4, 7, 15 and 30 days)/ revealed that the L_f magnitude of the false flocculation zone increased 5 to 6 times with the process of the culture's growth, also parallel with the increase in the concentration of the group precipitinogens.

Obviously, with the concentration of anatoxins, a partial freeing of specific antigen takes place from group precipitinogens and this leads to a shifting of the false zone of flocculation toward the actual one; consequently, the flocculation that started in the false zone overlaps the actual flocculation zone. In such case, only one, the initial flocculation is registered with the exaggerated values of L_f.

Hence, the results of the anatoxins' titration are influenced, according to the flocculation reaction, by the presence of bacterial precipitins in flocculent serums and by pertinent bacterial antigens in anatoxins.

The reactions formed between them are analogous to a specific reaction of anatoxin, but antitoxin distorts the results of the

latter. Consequently, in arranging the flocculation reaction one can only use such serums, which are freed from bacterial precipitins. According to our opinion, these serums can be obtained by inoculation of animals with antigen substances that contain no bacterial group precipitinogens, namely with preparations that are produced from a strain of other serological group than the strain used in the production of anatoxins, or, as is customary, from highly purified, but not crude, anatoxins.

For the same reason one should use a highly purified concentrated anatoxin as a standard antigen during titration of antitoxic serums according to the flocculation reaction.

Conclusions

1. Inasmuch as serums used in the flocculation reaction are obtained by inoculation of animals with antigen that has been accepted for the production of anatoxin, the results of the flocculation reaction, essentially specific, may become distorted on account of the concurrent reaction of group (bacterial) precipitinogens - precipitins.

2. Therapeutic, antitoxic, antibotuline serums can be used as flocculents, if they are purified and concentrated according to the "diaferm-3" method, and if they are not of agglutinating strain that has been accepted for the production of proper anatoxins. The ratio of EC/L_f to the serum in question should equal 1 even for least purified anatoxins.

3. The titer of botuline and tetanic anatoxins conforming to the flocculation reaction from the same flocculent serum depends upon a degree of the anatoxin's purity, namely on the antigen content

per 1 mg of common nitrogen.

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